

RESEARCH ARTICLE

Social synchronization of circadian locomotor activity rhythm in the fruit fly *Drosophila melanogaster*

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SUMMARY

Circadian clocks regulate the physiology and behaviour of organisms across a wide range of taxa. To keep track of local time, these clocks use a variety of time cues such as light–dark, temperature, food availability and social interaction cycles. This study assessed the role of social cues in modulating circadian clocks of the fruit fly *Drosophila melanogaster*. Using pair-wise interactions, we first estimated the percentage contribution of each interacting partner on the cumulative rhythmic behaviour of the pairs. Subsequently, we studied the effects of multi-individual (group-wise) interactions on the rhythmic behaviour of the group by estimating phase synchrony between individuals of different strains (having different circadian periods) maintained in both homogeneous and heterogeneous groups. Although it is known that social interactions improve synchrony between interacting individuals, we asked whether such interactions are able to synchronize the circadian rhythms of highly phase-desynchronized flies. We found that, although interactions between fly strains possessing different circadian periods failed to produce synchrony, social interactions among phase-desynchronized flies did enhance the phase synchrony of the interacting individuals. Differently phased individuals living in social groups displayed significantly greater phase synchrony than those living solitarily. Social synchronization is olfaction mediated as group-wise interactions among phase-desynchronized flies possessing compromised olfactory ability (*Or83b*⁰) did not improve phase synchrony. These results suggest that social cues synchronize the circadian clocks of *Drosophila* provided that the interacting individuals have similar clock periods.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/22/3742/DC1>

Key words: social, *Drosophila*, circadian, period, phase, olfaction.

INTRODUCTION

Cyclic social interactions have been shown to act as a zeitgeber (time cue) for the circadian clocks of many organisms, including honeybees, birds, bats, mice and fruit flies. Several early studies in mammals showed that the clock of the mother can set time in the offspring, especially during early development (Viswanathan and Chandrashekar, 1985; Reppert and Schwartz, 1986; Honma et al., 1987; Duffield and Ebling, 1998; Viswanathan, 1999). Social cues help in speeding up the rate of resynchronization to phase-shifted light–dark (LD) cycles. For example, in the diurnal rodent *Octodon degus*, resynchronization of circadian rhythms in females following a phase advance in LD cycles by 6 h was faster when females were paired with other females or males (Goel and Lee, 1997). Similarly, when hamster males were subjected to phase advancement in LD cycles by 8 h, their rhythms entrained faster when they were presented with females in estrous; however, mating with such females had a negative impact on the rate of re-entrainment (Honrado and Mrosovsky, 1989). In a separate study, it was shown that mice with variable circadian periods displayed synchronized behaviour as long as they were maintained together in a group (Crowley and Bovet, 1980). Similarly, members of a family of free-living beavers (*Castor canadensis*) displayed a synchronized period of ~27 h when confined as groups to caves in winters, only to return to a 24 h entrained rhythm in summers (Bovet and Oertli, 1974), suggesting that social cues maintain phase

synchrony between interacting individuals. By contrast, there are also a few reports of a lack of social synchronization of circadian rhythms. For example, in a study on hamsters, it was found that the locomotor activity rhythm of enucleated hamsters kept under LD cycles along with some normal-sighted individuals free-ran, whereas that of the sighted hamsters remained entrained to LD cycles (Refinetti et al., 1992). Likewise, in the sugar glider *Petaurus breviceps*, the rhythms of individuals maintained in pairs free-ran with different circadian periods (Kleinknecht, 2004), suggesting a lack of social synchronization. Similarly, in male rats, neither aggression by other males nor the act of mating with females caused a significant effect on circadian rhythms (Meerlo and Daan, 1998). Based on these studies, it is clear that the role of social cues in circadian timekeeping is far from being resolved.

The most conclusive evidence for social synchronization of circadian rhythms came from studies on honeybees, which showed that the overall activity–rest behaviour of bees within a colony results from a mutual (social) synchronization of the circadian rhythms of the individual honeybees (Frisch and Aschoff, 1987; Frisch and Koeniger, 1994). More recently, in a series of elegant studies, it was shown that social interactions in the fruit fly *Drosophila melanogaster* (which has a much lower degree of sociality compared with that of the honeybee) significantly altered the phase of the circadian clocks of interacting individuals (Levine et al., 2002; Krupp et al., 2008). It was reported that flies

kept in groups in an otherwise timeless (constant) environment exhibited a more coherent phase in their circadian activity–rest rhythm compared with that of those kept as isolated individuals. Furthermore, the authors showed that the overall phase synchrony of groups of rhythmic wild-type individuals decreased after they had interacted with arrhythmic loss-of-function *period* mutant (*per⁰*) visitors (Levine et al., 2002), suggesting that the circadian clocks that regulate locomotor activity rhythm in flies are modified by clocks of other individuals probably by means of social interactions. The authors went on to suggest that such an influence of social cues in altering clock properties was phase dependent. This was based on the results of an experiment in which visitor flies that were phase advanced (early) compared with the hosts were able to shift the phase of the host flies, whereas phase-delayed (late) visitors did not have a similar effect on the rhythms of the host (Levine et al., 2002). Finally, Levine and colleagues concluded that the effectiveness of social cues in shifting the phase of circadian clocks is time dependent. Furthermore, visitors with faster circadian clocks influenced the phase of individuals with slower clocks, whereas the latter did not modify the phase of individuals with faster clocks, suggesting that social cues act in a phase-dependent manner (Levine et al., 2002). This study also suggested that social cues that influence the circadian clock in *Drosophila* are communicated by means of chemosensory pathways as the phase synchrony of the circadian rhythms of *para^{sbl-1}* and *para^{sbl-2}* mutants that have decreased olfactory responses do not appear to be altered by the presence of arrhythmic visitors (Levine et al., 2002). In a similar and more recent study, it was shown that the presence of *per⁰* flies within a group of wild-type *CS* individuals (1:4 ratio) altered the transcript levels of core clock genes in the fly head and oenocytes of rhythmic wild-type flies and was accompanied by many behavioural changes (Krupp et al., 2008). Furthermore, these studies suggest that pheromone-producing oenocytes (Wigglesworth, 1970) have circadian clocks of their own and that release of certain pheromones is dependent on the central circadian clock (Krupp et al., 2008) and that oenocytes have a crucial role to play in the communication between peripheral oscillator(s) localized in the sensory system (Levine et al., 2002; Krupp et al., 2008).

Our studies differ from previous ones in the following respects. First, we verified the effect of pair-wise social interactions on the circadian rhythm of flies by comparing the results obtained from empirical studies of pair-wise interactions between flies with those from the analysis of theoretical time series data generated by simply pooling the time series obtained from two individual flies. We find that pair-wise social interactions did not affect the circadian clocks of flies except in those cases where a *per^L* mutant was involved in the interaction. We also studied the effect of group-wise interactions on the rhythmic behaviour of the group and estimated phase synchrony in individuals of different strains (having different circadian periods) maintained in both homogenous and heterogeneous groups. We find that group-wise social interactions between flies with different circadian periods do not affect the phase of their circadian rhythms. Although it is known that social interactions improve phase synchrony among socially interacting individuals (Levine et al., 2002), we asked whether such social interactions have the ability to synchronize highly phase-desynchronized flies (created by pooling flies from several out-of-phase LD cycles). The results showed that social interactions alter the phase of locomotor activity rhythm to cause greater phase synchrony among the socially interacting individuals.

MATERIALS AND METHODS

The general scheme followed in all experiments (with deviations as explained for specific experiments) was as follows: *Drosophila melanogaster* L. flies developed as pre-adults under 12h:12h LD cycles, and freshly emerged flies were collected and maintained in glass vials (length 95 mm, diameter 10 mm) as same-sex groups of 30–40 flies per vial. Flies were kept for the first 4 days under LD cycles and then transferred into constant darkness (DD), where they were maintained either in pairs or in groups, depending on the specific experiment.

Effect of pair-wise social interactions on the circadian locomotor activity rhythm

To study the effect of pair-wise social interactions on the circadian locomotor activity rhythm, we paired two individuals from the same or different strains in activity tubes, and recorded the locomotor activity behaviour of the pairs. From the activity data, we estimated their free-running period (τ) using ClockLab software (Actimetrics, Wilmette, IL, USA). We paired *per^S* ($\tau=18.77\pm0.31$ h; mean \pm s.d.), *per^L* ($\tau=28.96\pm0.77$ h) and *CS* ($\tau=23.58\pm0.57$ h) males in the following combinations: *per^S* + *per^L*, *per^S* + *CS* and *per^L* + *CS*. Furthermore, in order to generate flies with smaller period differences, we crossed *per^S*, *per^L* and *CS* flies in all combinations and used their virgin female offspring (because the *per* locus, being on the X chromosome, allows only female offspring to have two different alleles of *per*). The mean τ values of female offspring from crosses *per^L* \times *per^S* (*per^{LS}*), *per^L* \times *CS* (*per^{LC}*) and *per^S* \times *CS* (*per^{SC}*) crosses were 22.96 ± 0.21 h (mean \pm s.d.), 25.30 ± 0.61 h and 21.49 ± 0.50 h, respectively.

The locomotor activity behaviour of any given pair of flies comprising two different strains displayed the circadian period of both strains or of either strain, or arrhythmicity (supplementary material Fig. S1). We therefore estimated the number and hence percentage of pairs that showed a short period, long period, both short and long periods or arrhythmicity. It is possible that different periodicities might be detected merely owing to mixing of two sets of time series data with intrinsically different periods without any involvement of interaction between individuals. To address this possibility, first we separately recorded the locomotor activity behaviour of individual flies under DD for 10–12 days for each of the strains tested. For each strain, the time series data obtained from individual flies was tagged numerically (1–32). For each type of empirical pair-wise interactions between a given pair of strains, we artificially summed time series data by making pairs of time series having the same tag from two different strains. For example, time series data from individuals of the *per^S* strain were merged with time series data of individuals of the *per^L* strain (tag1 *per^S* + tag1 *per^L*; tag2 *per^S* + tag2 *per^L*, and so on). The summed time series data were used as a control (control-T). The percentage contributions of different patterns (short period, long period, both short and long periods or arrhythmicity) were estimated as described for empirically obtained time series data, and the relative distributions were compared for each set of empirically and mathematically summed data. To determine whether pair-wise social interaction has an effect on the circadian period of interacting flies, we compared period values of both the rhythmic components (short and long) between empirical and control-T data using ANOVA, where data type (empirical or control-T) and strain were treated as fixed factors. *Post hoc* multiple comparisons were performed using Tukey's test. All statistical analysis was carried out on STATISTICA for Windows release 5.0 B (StatSoft, 1995).

Effect of group-wise social interactions on the circadian locomotor activity rhythm

To study whether different strains of flies synchronize the circadian clocks of each other when maintained in groups, we took male flies from three strains – *per^S*, *CS* and *per^L* – and heterozygous females *per^{LS}*, *per^{LC}* and *per^{SC}* (produced by crosses between *per^S*, *per^L* and *CS*) and maintained them under LD cycles. After 4 days, we transferred the flies to DD and mixed flies of two different genotypes in equal proportions to form heterogeneous groups of 50–60 individuals in each vial (*per^S* + *per^L*, *per^S* + *CS* and *per^L* + *CS*, *per^{LS}* + *CS*, *per^{LC}* + *CS* and *per^{SC}* + *CS*) while homogeneous groups of flies (similar size) were maintained as controls. After 12 days, flies were separated, and their locomotor activity behaviour was recorded individually in DD. The phase of locomotor activity rhythm of flies on the day of separation was tracked by extrapolating backwards in time using the daily offset of locomotor activity rhythm as a phase marker. These phase values were used to draw circular diagrams. For statistical analyses of phase coherence, empirically obtained phase data were subjected to bootstrapping, where data were re-sampled with replacement to generate replicate sets of phase data (Good, 2005). For example, from a set of 'x' empirical data, we first generated a pool of 5x data, where each of the original data points was represented five times, and then from this pool each bootstrap sample of x values was randomly sampled five times, thus generating (N=5) replicates. These replicate phase values were subjected to circular vector analysis (Batschelet, 1981) to obtain magnitude (*r*, on a scale 0 to 1) and direction (*a*^o, on a scale of 1–360 deg or 1–24 h) of the phase-coherence vectors. A magnitude of 1 would mean that all individuals in a given set have exactly the same phase, whereas a magnitude of 0 would mean that individuals in the group are highly phase desynchronized. These data were used for ANOVA, where *r* and *a*^o values were treated as fixed factors and followed by *post hoc* multiple comparisons using Tukey's test.

Effect of group-wise social interactions on the circadian locomotor activity rhythm of phase-synchronized wild-type flies

To study the effect of long-term social interactions in groups on the circadian locomotor activity rhythm, we maintained *CS* flies in groups of 50–60 flies first under LD for 4 days and then in DD for 21 or 35 days. Another set of *CS* flies maintained solitarily first under LD for 4 days and then in DD for 21 or 35 days served as controls (supplementary material Fig. S2). Following this, the locomotor activity behaviour of both sets of flies – those maintained in groups or kept solitary – was monitored individually under DD. The phase of activity rhythm was estimated using regression lines drawn through the daily offsets of locomotor activity rhythm. The lines were extrapolated back to the last day of social interaction to obtain the phase of entrainment. The phase values thus obtained were bootstrapped with replacement as described above to obtain replicate data sets for the estimation of the magnitude and direction of phase-coherence vectors. These data were used for ANOVA, where *r* and *a*^o values were treated as fixed factors and followed by *post hoc* multiple comparisons using Tukey's test.

Effect of social interactions on the circadian locomotor activity rhythm of highly phase-desynchronized wild-type flies

Next, we asked whether social interactions are capable of bringing about phase synchrony among flies with a high degree of phase asynchrony. We performed two separate experiments to address this question. In the first experiment, we pooled flies from two separate LD cycles that were 10 h out of phase (LD cycles where lights were

on either during 10.00–22.00 h or 20.00–08.00 h), and, in the second, we pooled flies from six separate LD cycles (where lights were on during 05.00–17.00 h, 08.00–20.00 h, 11.00–23.00 h, 17.00–05.00 h, 20.00–08.00 h and 23.00–11.00 h). In both the experiments, we first maintained flies under various LD cycles for a period of 4 days and then pooled them in equal proportion from each LD cycle and transferred them to DD. In the first experiment, pooled flies from two opposite LD cycles were divided into two sets and transferred into DD, where one set was maintained for 21 days in groups of 50–60 flies per vial, whereas flies from the other set were kept solitary. In the second experiment, pooled flies from six different LD cycles were divided into six sets and transferred to DD; three sets were placed in groups of 50–60 flies per vial, whereas members of the remaining three sets were kept solitary (supplementary material Fig. S3). The locomotor activity behaviour of one set each of mixed and solitary flies was recorded after 2, 4 or 10 days to assess the extent of phase synchrony among individuals as a function of the number of days of social interaction. The phase of activity rhythm was estimated using regression lines drawn through the daily offsets of locomotor activity rhythm. The lines were extrapolated back to the last day of social interaction to obtain the phase of entrainment. The phase values thus obtained were bootstrapped with replacement to obtain replicate data sets for the estimation of magnitude and direction of phase-coherence vectors. These data were used for ANOVA, where *r* and *a*^o values were treated as fixed factors and followed by *post hoc* multiple comparisons using Tukey's test.

Role of olfaction in social interaction

Olfaction plays a crucial role in the social interactions in *Drosophila* (Levine et al., 2002; Krupp et al., 2008). Therefore, to study the role of olfaction in social interaction, we took flies with a loss-of-function mutation in the gene encoding *Or83b*, a receptor that is widely expressed in the olfactory circuit of the fly (Larsson et al., 2004). The *Or83b*⁰ flies were maintained in six different LD cycles, in the manner described above, pooled and divided into two sets and transferred to DD, each set having an equal contribution from all six LD cycles. One set of flies was maintained in groups of 50–60 flies per vial, whereas members of the other set were kept solitary. After 21 days, the locomotor activity behaviour of flies from both the sets was recorded individually under DD. To reconfirm the results, we backcrossed *w¹¹¹⁸* and *Or83b*⁰ flies to *CS* for six generations and then repeated the experiments. The phase of activity rhythm was estimated using regression lines drawn through the offsets of daily locomotor activity rhythm. The lines were extrapolated back to the last day of social interaction to obtain the phase of entrainment. The phase values thus obtained were bootstrapped with replacement to obtain replicate data sets for the estimation of the magnitude and direction of phase-coherence vectors. These data were used for ANOVA, where *r* and *a*^o values were treated as fixed factors and followed by *post hoc* multiple comparisons using Tukey's test.

RESULTS

Pair-wise social interactions after the circadian period of interacting flies only when *per^L* flies are involved

Percentage contributions of the interacting strains

The contribution of the phenotype of the rhythm (short period, long period, both short and long periods or arrhythmicity) from different combinations of strains of flies obtained empirically matched closely with control-T data for all pairs, except *per^S* + *per^L* (Fig. 1A). Thus pair-wise social interaction did not affect the

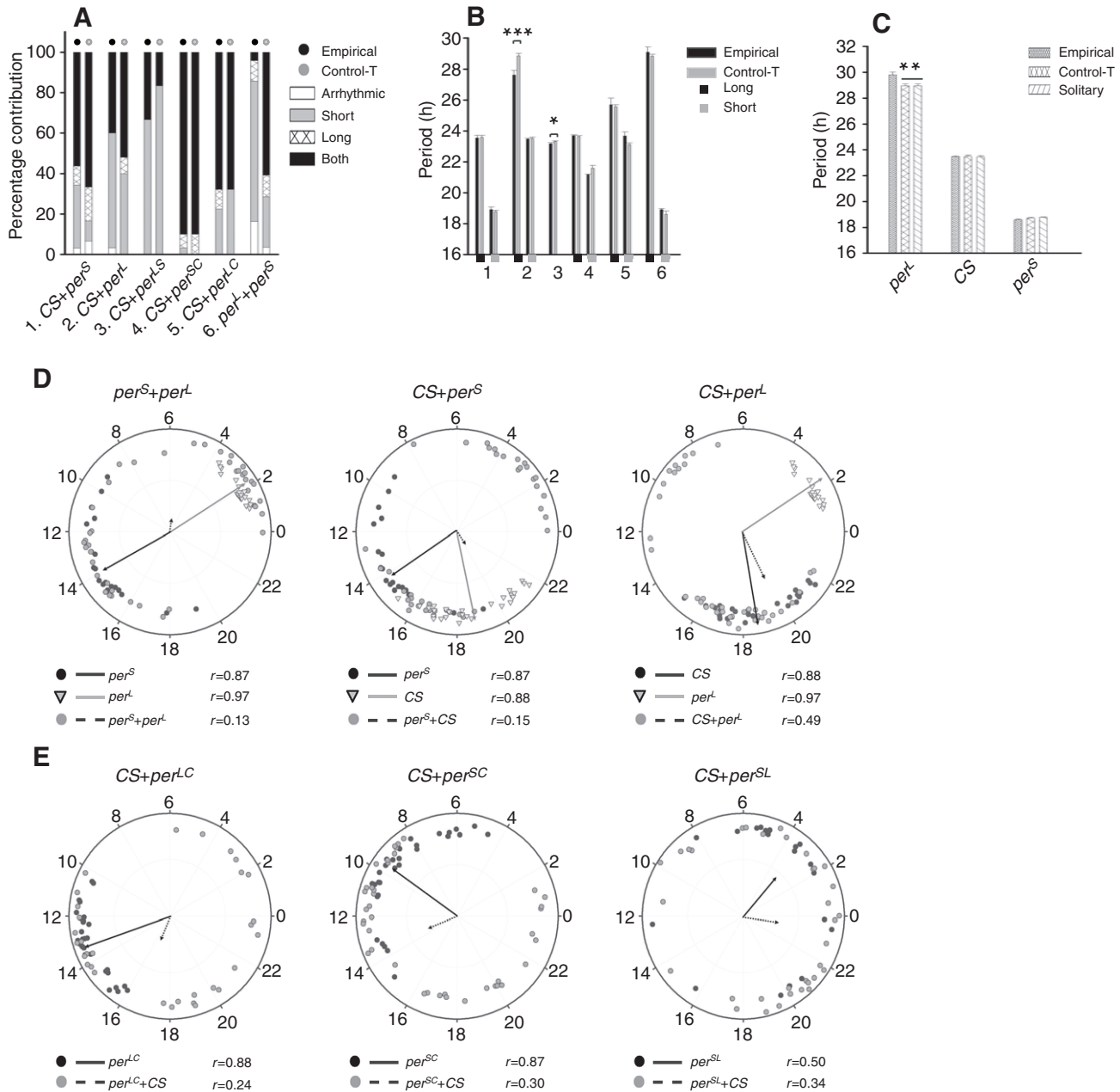


Fig. 1. (A) The percentage contributions of participating strains when allowed to interact socially one-on-one or pair-wise (empirical) and controls obtained theoretically by mixing time series data of interacting individuals (control-T). A black circle alongside the bars indicates that the distribution was obtained from empirical data, and a grey circle indicates that the distribution was obtained from control-T data. The percentage contribution of arrhythmic pairs is shown as the white shaded portion, and the presence of periodicities of both the interacting partners is shown by the black shaded portion. When only one period is detected, and the value of the period is that of the partner with the short period it is indicated by a grey shaded portion, whereas a cross-hatched portion indicates that the period of the partner with the long period is detected as a result of the theoretical or empirical mixing. (B) Effect of pair-wise social interaction on the circadian period of the pair. The genotypes of interacting pairs are indicated with numbers and correspond with those on panel A. The two sets of horizontal bars for each combination of strains give the value of mean circadian period of the long-period components (upper set, denoted by black square on the y-axis) or short-period component (lower set, grey square on y-axis) obtained empirically (black bars) or theoretically (grey bars). The *CS* + *per^{LS}* (3) combination has only one set of bars because the contributions of long (*CS*) and short (*per^{LS}*) components are not distinguishable (see text). Except in cases of *CS* + *per^L* (2) combination ($P < 0.0005$) and *CS* + *per^{LS}* (3) combination ($P < 0.05$) pairs, the interaction between no other strains caused a significant change in circadian period ($P > 0.05$) above what can be seen by theoretical mixing of time series (control-T). (C) The period values of homogenous pairs of only the *per^L* strain were significantly longer than those obtained from control-T data and solitary flies ($P < 0.005$). (D) Males maintained in heterogeneous groups have lower phase synchrony compared with that of homogenous controls. The symbols in the circular diagrams depict the phase (determined from activity offset). Black circles are phases of short-period strains, and grey triangles are those of long-period strains, whereas grey circles are those of individuals from mixed groups. Phase-coherence vectors are depicted as arrows, the length of which indicates the magnitude or extent of coherence and the direction of the mean phase of that phenotype (dark solid arrow, short-period strain; grey arrow, long-period strain; black dashed arrow, mixed group). (E) Similar to the top row, black circles and the black arrow represent the phase and coherence vectors of the females of the same strain group (but heterozygous at the *per* locus) control and solid grey circles and dashed black arrow represent values for a mixed group consisting of *CS* in combination with each of the heterozygous *per* mutant strains.

periodicity of the rhythm of the participating individuals (Fig. 1A), except when per^S and per^L flies are made to interact with each other. In this particular combination of strains, based on the empirical data, ~70% of the periodicities obtained from $per^S + per^L$ pairs displayed periods similar to per^S , ~4% similar to both, whereas the control-T data analysed similarly revealed a majority of time series having both periods (~61%), whereas ~25% of the periods were of the short type (Fig. 1A). In this combination alone, there is an apparent departure from theoretical pooling of time series such that per^S flies seem to influence the rhythm of their partner such that short-period rhythms dominate the locomotor activity behaviour of the pair.

Circadian period of the contributing strain

After estimating the percentage contributions of different strains of flies when socially interacting, we asked whether the circadian period of the singly rhythmic flies is altered owing to social interactions. The values of the circadian period obtained from empirical pairings were compared with that obtained theoretically (Fig. 1B) such that short-period values (empirical *versus* theoretical) and long-period values of each are compared for any significant change in period length. Except for per^L flies in the $per^L + CS$ combination, which showed shorter circadian period compared with that of controls ($P < 0.0005$), pair-wise social interaction did not have any effect on the circadian period of the interacting partners ($P > 0.05$). We were unable to assign distinct period values for the $per^{LS} + CS$ pairs because the circadian periods of both strains were indistinguishably close to each other; therefore, the data from this combination were excluded from the composite ANOVA and were analyzed separately. The results suggest that, when per^{LS} and CS flies were paired together, the empirically obtained period of the pairs was significantly shorter than that estimated from control-T data ($P < 0.05$) (Fig. 1B).

To study whether pair-wise social interaction among flies of the same strain alters the circadian period of their locomotor activity rhythm, we took per^S , per^L and CS flies and paired them with flies of the same strain, and recorded their locomotor activity behaviour under DD, and compared the circadian periods of the pairs with those obtained from the control-T data. The circadian period of per^L pairs was marginally, yet significantly, longer than that obtained from their theoretical controls ($P < 0.005$); however, the empirically obtained period values of CS or per^S pairs did not differ statistically from their respective controls ($P > 0.05$) (Fig. 1C). Thus, the ability of social interactions among pairs to alter the circadian period seems to be limited to that of some genotypes only – in this case, per^L , whereas the circadian clocks of most other strains and genotypes were not affected.

Social interactions among flies with varying period fail to produce phase synchrony in the group

As our studies showed very little effect of pair-wise interactions on the circadian clocks in flies, we examined the effect of social cues when flies are allowed to interact in groups of 50–60 individuals. Flies that intrinsically exhibit very different circadian periods (CS, per^S , per^L , per^{LC} , per^{SC} and per^{LS}) were maintained in groups of 50–60 flies per vial. Flies were kept as experimental (mixed group or heterogeneous) and control (same strain or homogenous) groups for a period of 12 days, following which they were separated and their locomotor activity behaviour was recorded individually, and the phase of locomotor activity rhythm was then determined. We find that the heterogeneous groups $per^S + per^L$, $per^S + CS$, $per^L + CS$, $per^{LC} + CS$, $per^{SC} + CS$ and $per^{LS} + CS$ of flies had significantly lower phase synchrony than their respective homogeneous controls

($P < 0.0005$; ANOVA followed by Tukey's test) (Fig. 1D, black dashed arrows and circles).

Furthermore, we found that their phase synchrony was either similar to or lower than that estimated for simulated (mathematically pooled) heterogeneous controls (data not shown). These results indicate a lack of phase synchrony among socially interacting flies with variant circadian periods.

Group-wise social interactions in phase-synchronized flies enhance phase synchrony of the group

Based on previous studies that showed that the phase synchrony among socially interacting flies when allowed to interact for 2 weeks under DD is higher than that of solitary controls, we conducted an assay with wild-type CS flies to determine whether such synchrony persists for longer durations (up to 21 or 35 days). Freshly emerged CS males were maintained under LD cycles for 4 days, then divided into two groups and transferred into DD. Flies from the first group were isolated and kept solitary, whereas those from the second group were maintained in groups of 50–60 individuals per vial. After 21 or 35 days, the locomotor activity behaviour of flies maintained solitarily and in groups was recorded individually in DD and their phase coherence compared. The results showed that the phase synchrony of flies maintained in groups for 21 days ($P < 0.0005$) or 35 days ($P < 0.001$) was significantly greater than their respective solitary controls (Fig. 2A) thus indicating that group level interactions enable flies to influence the circadian clocks of each other such that they remain more in-phase compared to singly housed individuals.

Group-wise social interactions enhance phase synchrony of highly phase-desynchronized flies

The above study showed us that group-wise social interactions in CS flies resulted in enhanced phase synchrony in flies that had previously all been under same environmental regime and would therefore be expected to have fairly synchronized clocks to begin with. Therefore we examined whether flies with a high degree of phase desynchrony can achieve synchrony via social interaction. We first desynchronized the phase of locomotor activity rhythm of CS flies by keeping them under two oppositely phased LD cycles (with lights-on between 10.00–22.00 h and 20.00–08.00 h) for a period of 4 days, then divided them into two sets, and transferred to DD. Individuals from the first set were maintained solitary, while those from the second set were mixed, taking equal number of flies from both LD cycles, to form composite groups of 50–60 individuals per vial. After 21 days, locomotor activity behaviour of flies from both the sets was recorded individually under DD. Analysis revealed that magnitude of phase-coherence vector of flies maintained in groups was significantly greater than those kept solitary ($P < 0.05$) (Fig. 2B). Rayleigh's test showed that the phase coherence of flies living solitarily ($P = 1.00$) and in a group ($P = 0.20$) did not differ significantly; however, the level of phase synchrony among flies living in groups was significantly greater than that of those living solitarily (Fig. 2B). This suggests that flies living in groups are able to synchronize each other's circadian rhythms.

To create even greater phase asynchrony among interacting flies than achieved above ($r = 0.0004$), we next maintained CS flies under six different LD cycles and then pooled them together, with equal contribution from each LD cycle to form six sets. Three sets of flies were kept in groups of 50–60 individuals per vial, and the remaining three sets were kept solitary. To assess the time-course of achievement of phase coherence, the three sets of flies kept in groups under DD were allowed different durations of social interaction.

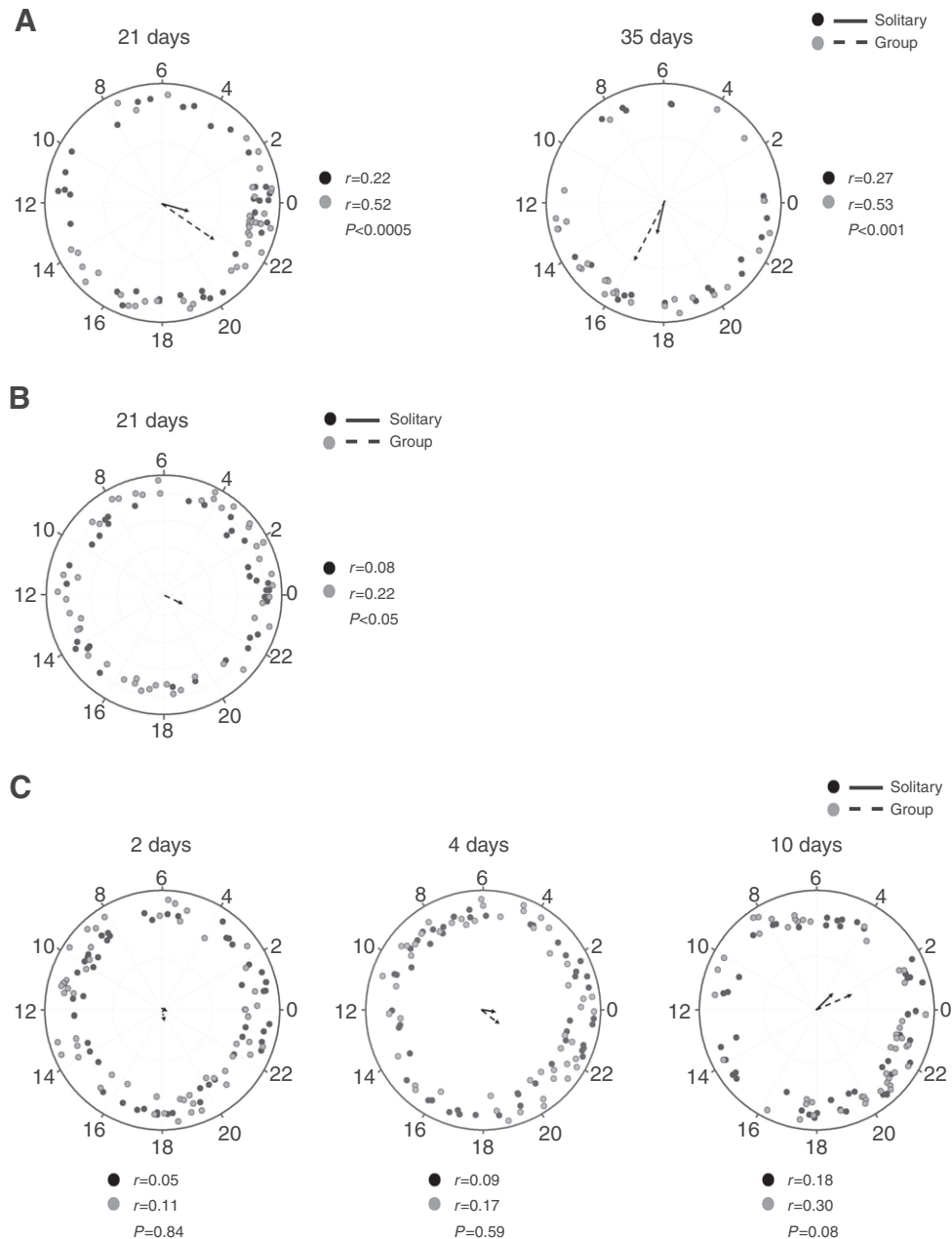


Fig. 2. Group-wise social interactions enhance phase synchrony. (A) The phase of activity rhythm of individual flies previously kept in groups under light–dark (LD) cycles and then in constant darkness (DD) for prolonged durations shows greater phase synchrony when compared with that of isolated individuals. At 21 days (left) in constant darkness (DD), the activity rhythms of flies in groups (grey dots) are more synchronous than those of their isolated controls (black dots, $P<0.0005$). A similar difference is evident at 35 days (right) ($P<0.001$). (B) Flies that were first entrained to two LD cycles of opposite phase (lights on 10.00–22.00 h or 20.00–08.00 h) were allowed to interact in groups under DD and show greater synchrony compared with that of their isolated controls ($P<0.05$). (C) Flies first entrained to six different LD cycles were allowed to interact for either 2, 4 or 10 days under DD. Compared with solitary controls, the synchrony of flies maintained in groups is greater after 2 days ($P=0.84$), 4 days ($P=0.59$) and 10 days ($P=0.08$) of group living. The black circles and arrow represent the phase (offset) and phase-coherence vector of isolated individuals, whereas the grey circles and dashed black arrow denote the phase of individuals in groups.

The members of the group were separated after 2, 4 or 10 days of interaction to determine the phase of the locomotor activity rhythms of individual members of the group under DD. The effect of group living did not have any measurable effect on the phase synchrony of interacting individuals after 2 days ($P=0.84$) or 4 days ($P=0.59$); however, after 10 days, differences between the phase synchrony of flies living in groups and living solitary became greater, although it did not reach statistically significant levels ($P=0.08$). Rayleigh's

test showed that the phase-coherence vector did not possess any direction in flies living solitary ($P>0.05$). However, in flies maintained as groups, although the direction of the phase-coherence vector indicated a uniform distribution after 2 or 4 days of social interaction ($P>0.05$), after 10 days of social interaction, it became significantly non-uniform ($P<0.01$) (Fig. 2C). This suggests that increasing the duration of social interactions enhances phase synchrony among the members.

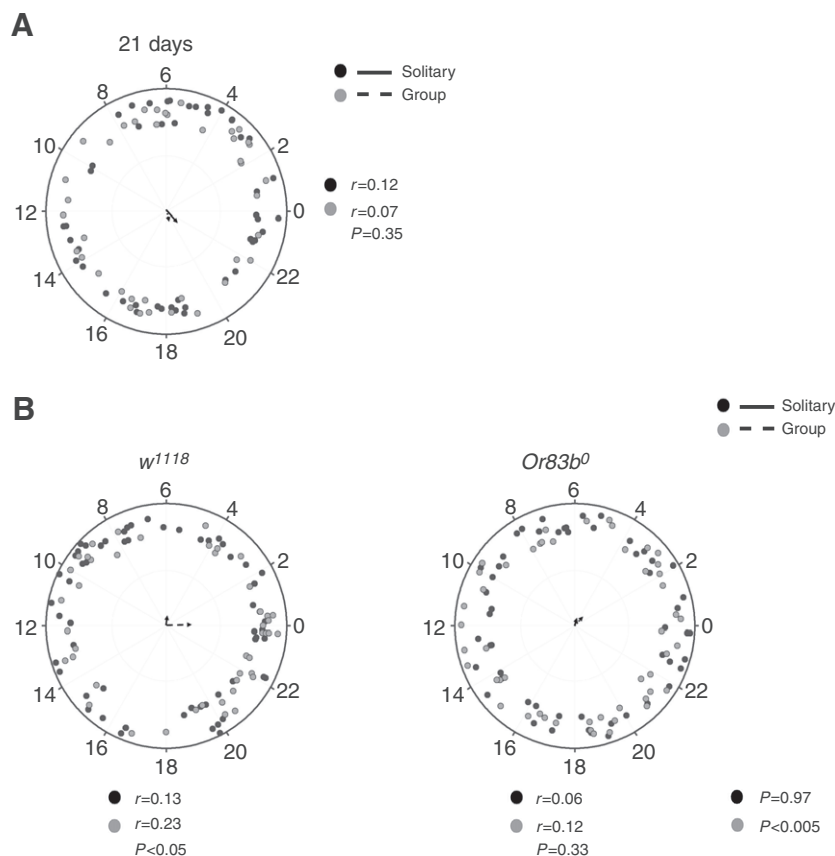


Fig. 3. Social interactions require a functional olfactory ability. Flies that were desynchronized by entraining them to six different light–dark (LD) cycles were pooled and kept in constant darkness (DD) to enable social interactions. (A) No difference in phase coherence is seen between flies maintained in groups compared with isolated individuals ($P=0.35$) when *Or83b⁰* strains are assayed after 21 days in DD. (B) *Or83b⁰* and *w¹¹¹⁸* flies backcrossed to *CS* for six generations then subjected to the above protocol and assayed after 12 days revealed that synchrony of group-living *w¹¹¹⁸* flies is significantly greater ($P<0.005$) than that of group-living *Or83b⁰* flies, whereas there is no difference in synchrony of isolated individuals from both groups ($P=0.97$). The black circles and arrow represent the phase (offset) and phase-coherence vector of isolated individuals, whereas the grey circles and dashed black arrow denote the phase of individuals in groups.

Lack of functional olfactory ability lowers the phase synchrony of flies in groups

As we found that social interactions were capable of synchronizing the phase of the circadian activity rhythm, we asked whether this might be mediated through olfactory signals. We took flies with a loss-of-function mutation in the widely expressed olfactory receptor *Or83b* (designated *Or83b⁰*) and placed them in six different LD cycles (with lights on during 05.00–17.00 h, 08.00–20.00 h, 11.00–23.00 h, 17.00–05.00 h, 20.00–08.00 h and 23.00–11.00 h) for 4 days, and then pooled them in equal proportion from each LD cycle and then divided these highly phase-desynchronized flies into two sets. While both sets were transferred simultaneously to DD, flies from the first set were kept solitary, and those from the second set were housed in groups of 50–60 flies per vial. After 21 days, the locomotor activity behaviour of flies was recorded individually in DD. The phase coherence of flies living in groups did not differ statistically from that of those living solitary ($P=0.35$) (Fig. 3A).

We repeated this experiment by using *Or83b⁰* and *w¹¹¹⁸* flies that had both been backcrossed into the *CS* background for six generations. This served to eliminate any effects of the genetic background of the null mutant that could intrinsically cause lower levels of synchronization that might be independent of the olfactory dysfunction. The phase synchrony of *w¹¹¹⁸* flies living in groups was significantly greater than that of *Or83b⁰* flies in groups ($P<0.005$), whereas the phase synchrony of flies of both strains living solitary did not differ ($P=0.97$) (Fig. 3B), suggesting that social synchronization in fruit flies requires a functional olfactory ability.

DISCUSSION

We have used two types of protocols to address the question of whether social cues can influence the circadian clocks in fruit flies,

one of which was similar to that employed by Levine and colleagues (Levine et al., 2002), where flies were synchronized initially under one type of LD cycle and then allowed to interact pair-wise or in groups (up to 60 individuals) under constant darkness, following which we compared the level of synchrony among control and experimental groups. The results of our study suggest that flies failed to synchronize the circadian rhythms of each other under DD when social interactions were limited to occur between two individuals, as we found that the circadian period of interacting individuals did not differ from theoretical controls (Fig. 1), except in the case when such interactions took place between *per^L* and wild-type, or *per^{LS}* and wild-type, flies. However, when wild-type *CS* flies were made to interact socially in groups of 50–60 individuals, flies living in groups were found to display significantly higher phase synchrony compared with that of those living solitary, confirming the findings of Levine and colleagues (Levine et al., 2002) (Fig. 2A). When flies of different strains, differing in circadian period, were maintained in heterogeneous groups, phase synchrony of the group was reduced compared with that of controls, suggesting that the rhythms of flies drift away each other when individuals of the interacting group have different circadian periods owing to lack of social synchronization (Fig. 1D). In the second protocol, flies pooled from many differently phased LD cycles were used to examine whether social cues can improve the synchrony of highly desynchronized flies. *CS* flies that originated from a highly desynchronized set when allowed to interact in groups displayed significantly greater phase synchrony than that of solitary controls. Furthermore, our study showed that flies with compromised olfactory ability (*Or83b⁰*) failed to influence the circadian rhythms of each other, resulting in a lack of phase synchrony (Fig. 3), suggesting that social synchronization among flies requires a functional olfactory ability. Thus, using very

different experimental approaches, we confirm that social cues synchronize circadian locomotor activity rhythm, provided that the circadian periods of the interacting flies are similar.

Previous studies have suggested that circadian clocks of the fruit fly *D. melanogaster* are sensitive to social cues that modulate the clock phase (Levine et al., 2002). Other organisms such as mice and beavers show an effect of social interactions on the circadian period (Halberg et al., 1954; Bovet and Oertli, 1974; Crowley and Bovet, 1980). Our study showed that social interactions among flies with different periods did not cause a measurable change in circadian period when made to interact one-on-one, except when *per^S* and *per^L* flies were paired together (Fig. 1A). Such flies failed to synchronize the circadian clocks of each other even in group-wise social interaction (Fig. 1D), which might be due to the phases of their circadian rhythms gradually drifting away from each other. However, when phase-desynchronized wild-type *CS* flies were made to interact socially, their phase synchrony was significantly enhanced. These results suggest that social cues are able to synchronize circadian clocks when the periods of the interacting flies are similar, implying that social interactions are relatively weak synchronizing cues for *Drosophila* circadian clocks.

Social synchronization alters the circadian period of interacting flies only in cases when the *per^L* strain was involved either directly or indirectly (Fig. 1B,C); the circadian period of *per^L* and *per^{LS}* flies was significantly different from controls – however, no such effect was evident in pairs without *per^L* flies, suggesting that *per^L* strains of *Drosophila* are sensitive to social cues. These results are consistent with the findings of a previous study by Crowley and Bovet (Crowley and Bovet, 1980), wherein the phase synchrony of mice living in groups was found to be achieved by period changes. Furthermore, in a previous study, Levine and coworkers (Levine et al., 2002) showed that the clocks of *per^L* host flies were responsive to *per^S* visitors, whereas *per^L* visitors were unable to bring about significant change in the phase synchrony of *per^S* hosts. It is likely that the general nature of slower circadian clocks in the *per^L* flies would make them more sensitive to social cues compared with their faster interacting partners (through some unknown mechanisms). This is because *per^L* flies will invariably lag behind the faster interacting partners in terms of their activity–rest or sleep–wake cycles. It has been shown in previous studies that early visitors have significant impact on the phase synchrony of late hosts, whereas early hosts are not affected by late visitors (Levine et al., 2002), suggesting that delayed or late clocks are socially more responsive. Social cues were probably effective owing to the modulation of clock gene expression, which has impact on the profile of pheromones secreted by the oenocytes and hence social behaviour (Krupp et al., 2008).

Previous studies have shown that, in the event of the absence of potential zeitgebers, social cues serve as time cues for *D. melanogaster* circadian clocks (Levine et al., 2002). This is evident in our study also – wild-type flies, whose phase was already synchronized by entrainment to LD cycles, maintained together as groups for 21 or 35 days under DD had significantly greater phase synchrony than those kept solitary (Fig. 2A), suggesting that social interactions enable synchronization of circadian clocks. The study by Levine and colleagues (Levine et al., 2002) did not answer the question of whether phase synchrony in socially interacting flies was due to active manipulation of phase or due to similarity in their phase as all the flies had previously been entrained to the same LD cycle. We addressed this by allowing highly phase-desynchronized flies to interact in social groups (Fig. 2B,C). Flies with a high degree of phase asynchrony show improved phase synchrony after as few

as 10 days of group living (Fig. 2C), suggesting that social cues can act as a zeitgeber for the circadian clocks of the fruit fly *D. melanogaster* when sufficiently large number of flies with similar clock periods are allowed to interact.

From previous studies, we know that olfaction plays a major role in communication during social interactions (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008). To examine this critically, we took flies with a loss-of-function mutation for a widely expressed olfactory receptor *Or83b* (*Or83b⁰*), which is known to disrupt the response to many odors (Larsson et al., 2004), and allowed them to interact socially under DD for 21 days. Socially interacting *Or83b⁰* flies had extremely poor phase synchrony (Fig. 3A), confirming that social interaction in *Drosophila* is olfaction mediated. Furthermore, when *Or83b⁰* and *w¹¹¹⁸* flies backcrossed to *CS* flies for six generations were used, the phase synchrony of *w¹¹¹⁸* flies was significantly greater than that of *Or83b⁰* flies ($P < 0.005$) (Fig. 3B,C). Thus, these results suggest that social cues play a significant role in enhancing phase synchrony among flies living in groups and that olfaction is the likely mode of communication.

Our study suggests that pair-wise social interactions among flies do not alter the circadian locomotor activity rhythm, except when flies from the *per^L* strain were involved as the interacting partner (Fig. 1A–C). Similarly, social cues failed to evoke synchrony in heterogeneous (mixed) social groups when two strains of flies with different circadian periods were made to interact. Long-term group-wise social interactions among highly phase-desynchronized wild-type flies enhanced phase synchrony of the group compared with that of solitary individuals (Fig. 2). Social communication among individuals living in groups is olfaction mediated (Fig. 3). The results of our study indicate that the fruit fly *D. melanogaster* use phase changes and not period changes to attain synchrony among socially interacting individuals. Social interactions might be of great advantage to organisms living in groups as it maximizes synchrony and hence the likelihood of most members taking part in community activity, which is crucial for enhancing fitness of the society. However, our study suggests that social interactions alone might not be sufficient to entrain the circadian clocks of *Drosophila*.

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